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ADVANCED BIOMEDICINE
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USER MANUAL

Genequality
AZF MX

Code 04-23A
Code 04-23R

Kit for the identification of deletions in the AZF locus

Compliant to the European Guidelines
"EAA/EQMN best practice guidelines for molecular diagnosis of Y-chromosomal microdeletions. State of the art 2004"
Simoni M., Bakker E., Krausz C. *Int. J. Andrology*, 2004, 27: 240-249.

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IVD

AZF MX 04-23_MANUAL_eR250111

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1 PRODUCT INFORMATION

1.1 Intended Use

The GENEQUALITY AZF-MX kit is an IVD for the identification of deletions in the AZF locus, involved in male infertility

This user manual describes the instructions for use of the following products:

GENEQUALITY AZF-MX
Includes all the reagents for the amplification and visualization by agarose gel electrophoresis.

Code	Product	Pkg
04-23A-12	GENEQUALITY AZF-MX	12 tests
04-23A-25	GENEQUALITY AZF-MX	25 tests

GENEQUALITY AZF-MX – amplification reagents
Includes all the reagents for the amplification.

Code	Product	Pkg
04-23R-12	GENEQUALITY AZF-MX – amplification reagents	12 tests
04-23R-25	GENEQUALITY AZF-MX - amplification reagents	25 tests

2 KIT CONTENT

Attention: the different contents correspond to different kits.
(legend: **X** = component included in the kit; **0** = component not included in the kit)

BOX P

STORE AT – 30 °/– 20 °C

code 04-23A	code 04-23R	DESCRIPTION	LABEL	TUBE (T) OR LID COLOUR	12 tests	25 tests
X	X	Single dose premixed tubes for Multiplex I.		Clear (T)	12	25
X	X	Single dose premixed tubes for Multiplex II.		Blue (T)	12	25
X	X	Single dose premixed tubes for Multiplex III.		Yellow (T)	12	25
X	X	Thermostabile Hot-start Taq DNA polymerase	Super AB Taq 5 U/µL	Red	25 µL	50 µL

SMALL BAG

STORE AT – 30 °/– 20 °C

code 04-23A	code 04-23R	DESCRIPTION	LABEL	TUBE (T) OR LID COLOUR	12 tests	25 tests
X	X	Reference DNA (from an undeleted male)	Reference DNA (undeleted male)	Blue	1X 20 µL	1X 40 µL

BOX F

STORE AT +2°/ +8°C

code 04-23A	code 04-23R	DESCRIPTION	LABEL	TUBE (T) OR LID COLOUR	12 tests	25 tests
X	0	Electrophoresis loading buffer (6X solution)	Bromophenol blue	Blue	200 µL	350 µL
X	0	Ethidium Bromide solution (2.5 mg/mL)	Ethidium Bromide TOXIC R 23 68 S 36/37 45	Red	100 µL	200 µL
X	0	DNA Molecular Weight Marker (MW)	MW Marker	Yellow	100 µL	200 µL

BOX A

STORE AT +15°/ +25°C

code 04-23A	code 04-23R	DESCRIPTION	LABEL	TUBE (T) OR LID COLOUR	12 tests	25 tests
X	0	Agarose molecular biology grade	AGAROSE		1X 10 g	1X 20 g
X	0	Electrophoresis buffer TRIS-Acetate -EDTA pH 8.0	50 X TAE		1X 40 mL	1X 70 mL

3 STORAGE AND STABILITY OF THE REAGENTS

Each component of the kit must be stored according to the directions indicated on the label of the single boxes.
In particular:

Box P	Store at -30°/ -20°C
Small bag	Store at -30°/ -20°C
Box F	Store at +2/+8°C
Box A	Store at +15/+25°C

When stored at the recommended temperature, all test reagents are stable until their expiration date, indicated on the labels.

4 PRECAUTIONS FOR USE

- The kit must be used only as an IVD and be handled by qualified investigators, who are educated and trained in molecular biology techniques applied to diagnostics;
- Before starting the kit procedure, read carefully and completely the instruction manual.
- Keep the product out of heating sources;
- Do not use any part of the kit if over the expiration date;
- In case of any doubt about the storage conditions, box integrity or method application, contact AB ANALITICA technical support at: laboratorio@abanalitica.it before using the kit.

In the amplification of nucleic acids, the investigator has to take the following special precautions:

- Use filter-tips;
- Store the biologic samples, the purified DNA, the reference DNA included in the kit and all the amplification products in different places from where amplification reagents are stored.
- Organise the space in different pre- and post-PCR units; do not share consumables (pipets, tips, tubes,…) between them.
- Change frequently the gloves;
- Wash the bench surfaces with 5% sodium hypochloride;
- Store the extracted DNA at +2°/ +8°C for short term, or freeze them at -30°/ -20°C for long term storage.
- Thaw the PCR premixes at room temperature before use. Add Taq DNA polymerase and purified DNA very quickly at room temperature, better if in an ice-bath.

5 SAFETY RULES

5.1 General safety rules

- Wear disposable gloves to handle reagents and clinical samples, wash your hands at the end of work.
- Do not pipet with mouth.
- Since no known diagnostic method can assure the absence of infective agents, it is a good rule to consider every clinical sample as potentially infectious and handle it as such.
- All the devices that get directly in touch with clinical samples should be considered as contaminated and disposed as such. In case of accidental spilling of the samples, clean up with 10% Sodium Hypochloride. The materials used to clean up should be disposed in special containers for contaminated products
- Clinical samples, materials and contaminated products should be disposed after decontamination by:

  immersion in a solution of 5% Sodium Hypochloride (1 volume of Sodium Hypochloride solution every 10 volumes of contaminated fluid) for 30 minutes

  OR

  autoclaving at 121°C at least for 2 hours (NOTE: do not autoclave solutions containing Sodium Hypochloride!!)

5.2 Safety rules about the kit

The risks for the use of this kit are related to the single components:

Dangerous components:

ETHIDIUM BROMIDE (included in 04-23C and 04-23A)
3,8-diamino-1-ethyl-6-phenylphenantridiumbromide (Ethidium Bromide) <2%

Description of risk: T (Toxic)

RISK SENTENCES AND S SENTENCES

R 23 and R 68	Toxic for inhalation. Risk of irreversible effects.
S 36/37 45	Wear laboratory coat and disposable gloves. In case of accident or discomfort, seek for medical assistance and show the container or label.

R and S sentences refer to the concentrated product, as provided in the kit.

In particular for Ethidium Bromide, until the dilution in the agarose gel.

In manipulating concentrated Ethidium Bromide, use a chemical dispensing fume cabinet. Always wear disposable gloves and laboratory coat in manipulating the diluted Ethidium solution as well.

The product can not be disposed with the common waste. It must not reach the drainer system. For the disposal, follow the local law.
In case of accidental spilling of Ethidium Bromide, clean with Sodium hypochloride and water.

Material safety data sheet (MSDS) of the kit is available upon request.

6 MATERIALS REQUIRED, BUT NOT PROVIDED

6.1 Reagents

- Reagents for DNA extraction
- Sterile DNase and RNase free water;
- Distilled water;
- Reagents for agarose gel electrophoresis (necessary for code 04-23R)

6.2 Instruments

- Laminar flow cabinet (use is recommended while adding TAQ polymerase to the amplification premix to avoid contamination; it would be recommended to use another laminar flow cabinet to add the extracted DNA);
- Micropipettes (range: 0.2-2 µL; 0.5-10 µL; 2-20 µL; 20-200 µL; 100-1000 µL);
- Thermal cycler;
- Microcentrifuge (max 12-14,000 rpm);
- Balance;
- Vortex;
- Magnetic heating stirrer or microwave.
- Chemical cabinet (its use is recommended in handling Ethidium Bromide);
- Horizontal electrophoresis chamber for agarose minigel;
- Power supply (50-150 V);
- UV Transilluminator;
- Photo camera or image analyzer.

6.3 Materials

- Disposable gloves;
- Disposable sterile filter-tips (range: 0.2-2 µL; 0.5-10 µL; 2-20 µL; 20-200 µL; 100-1000 µL)
- Graduate cylinders (1 L) for of TAE dilution;
- Pyrex bottle or Becker for agarose gel preparation;
- Parafilm.
- Microtubes (1.5 – 2.0 mL)

7 PREPARATION OF THE REAGENTS

Preparation of 1 L of 1X TAE buffer:

Mix 20 mL of 50X TAE with 980 mL of distilled water.

8 INTRODUCTION

Nowadays, the analysis of microdeletions of Y chromosome is considered an essential diagnostic approach to study male infertility.
Recent studies have divided the Y chromosome long arm into three regions, called AZF (Azoospermia Factors), frequently found to be deleted in some azoospermatic and serious oligozoospermatic subjects.
These loci (**AZFa, AZFb** e **AZFc**) contain genes that control the correct course of spermatogenesis (Fig.1) Alterations in one or more AZF loci cause a drastic reduction of germinal cells up to their complete absence.
Several studies marked the relation between the deletions and the presence of repeated DNA sequences showing that a recombinational event between the two proviral sequences is the basis of AZFa deletion (Kamp et al., 2000; Sun et al., 2000).
AZFc deletions are generally more uniform and follow the recombination between blocks of repeated DNA sequences flanking the region. (Kuroda-Kawaguchi et al., 2001).
A diagnosis of Y-related infertility is suspected in males with serious Azoospermia or Oligozoospermia, associated with anomalous morphology and/or spermatic motility, in absence of other known causes. In 5 to 10% of these subjects microdeletions of Y chromosome are identified by molecular analysis.
To evaluate the integrity of Y euchromatic region, at least four genes should be investigated: **USP9Y (or DFFRY), DBY, RBMY and DAZ**. These genes are currently considered to be directly or indirectly involved in male fertility.

USP9Y is a single copy gene located in AZFa region, it has its homologous on the X chromosome and it is expressed ubiquitously. (Brown et al., 1998).
DBY, located in AZFa region, is a single copy gene with an homologous on the X chromosome as well, but it gives a specific transcript at the testicular level (Lahn & Page, 1997).
RBMY is a multicopy gene mainly located in AZFb region and expressed exclusively at testicular level (Ma et al, 1993).
DAZ is a gene located in AZFc region in four copies arranged in two clusters (Saxena et al., 2000).

Recently, PCR amplification of short DNA sequences (STS) within AZF loci has been recognised as the best method to detect microdeletions in these regions.

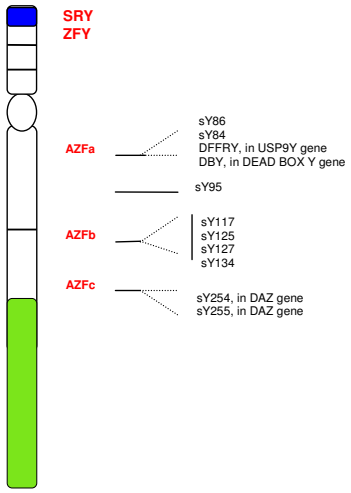

Fig.1:
SCHEMATIC VISUALIZATION OF Y CHROMOSOME AND AZF LOCI
Markers for the detection of microdeletions in AZF locus.

Published data demonstrated that the routinely performed diagnostic protocols were very different and often leading to inaccurate or incomplete diagnosis. The need of standardization was highlighted in a preliminary publication of the European Guidelines that described the main characteristics of the amplification reaction and the markers required to have a complete and reliable diagnosis (Simoni et al., 1999).
Four years after this preliminary publication, the complete sequencing of Y chromosome (Skaletsky et al., 2003) and the knowledge about the molecular mechanisms of deletions (Kampo et al., 2000; Sun et al., 2000; Repping et al., 2002) the **European Guidelines** were published **(Simoni et. Al., 2004).**
The European Guidelines give precise indications for the selection of the patients to be screened. The authors suggest.

- A multiplex PCR amplification of genomic DNA must be performed.
- The amplification of the ZFX/ZFY gene is an appropriate internal PCR control because the primers amplify a unique fragment both in male and female DNA, respectively.
- The basic characteristics of the selected markers are: Y-specificity, absence of other homologous sequences, non-polymorphic.

- At least two loci in each AZF region should be analysed:
  for AZFa: sY84, sY86
  for AZFb: sY127, sY134
  for AZFc: sY254, sY255

- The SRY gene should be included in the analysis as a control for the testis-determining factor on the short arm of the Y chromosome and for the presence of Y-specific sequences when the ZFY gene is absent (e.g. in XX males).

9 TEST PRINCIPLE

PCR (**P**olymerase **C**hain **R**eaction) was the first method of DNA amplification described in literature. (Saiki RK et al., 1985). It can be defined as an *in vitro* amplification reaction of a specific part of DNA (target sequence) by a thermo-stable DNA polymerase.

In the reaction, three segments of nucleic acid are involved: the double strand DNA template to be amplified (target DNA) and two single-strand oligonucleotides “primers” that anneal in a specific way to the template DNA.
The DNA polymerase begins the synthesis process at the region marked by the primers and synthesizes new double stranded DNA molecules, identical to the original double stranded target DNA region, by facilitating the binding and joining of the complementary nucleotides that are free in solution (dNTPs). After several cycles, we can get millions of DNA molecules which correspond to the target sequence.
The sensitivity of this test makes it particularly suitable to the application in laboratory diagnostic.

The **multiplex** amplification allows the simultaneous amplification of different DNA sequences in the same reaction, by mean of a selected primers mix.

10 PRODUCT DESCRIPTION

The amplification of short DNA sequences (STS) in AZF loci is the best method to verify the presence of microdeletions in Y chromosome.
In particular, the strategy of the proposed method consists of the amplification of eleven markers by mean of 3 multiplex PCR.
AZF MX method is compliant to the indications of the recently proposed European Guidelines for diagnostic testing of Y-chromosomal microdeletions (Simoni M et al., 2004) that define the basic characteristics of the primer set and of the amplification protocol to enable the detection of almost all the clinically relevant deletions.

The amplification of the internal control (in the ZFX/ZFY gene) allows to verify the good quality of the extracted DNA and, at the same time, the absence of amplification inhibitors, avoiding false positive results.

The kit includes reference DNA from a non-deleted male. The amplification of the reference DNA (with the presence of all the expected bands) is a guarantee of a correct course of the reaction.
The reference DNA is of human origin but it is not dangerous for the operator.

The kit is in premix format: all the reagents for the amplification are pre-mixed and aliquoted in monodose test tubes to which Taq polymerase and the extracted DNA will be added.

This premix format allows the reduction of the manipulation in pre-amplification steps, with a considerable time saving for the operator, the repeated freezing/thawing of reagents (that could alter the product performances) is avoided and, above all, this form reduces at minimum the risk of sample contamination and the risk to get false positive results.

Nevertheless, it is always recommended to use all the proper amplification controls.

11 COLLECTION, MANIPULATION AND PRE-TREATMENT OF SAMPLE

The procedure for the analysis of microdeletions of Y chromosome starts from the collection of whole blood samples.

The sample collection should follow all the usual sterility precautions.

Blood should be treated with EDTA. Other coagulating agents, as heparin, are strong inhibitors of TAQ polymerase and so they could alter the efficiency of the amplification reaction.

Fresh blood can be stored at +2 / +8°C for short time; if DNA is not extracted shortly, it is necessary to freeze the sample.

12 PROCEDURE

12.1 DNA extraction

Any DNA extraction method can be used, provided that allows the extraction of pure and integral DNA.
Extracted DNA should be quantified by spectrophotometric measurement.
The amount of DNA to be used in the amplification is 180 ng, therefore, the purified DNA solution should be diluted taking into account that the available volume in each premix tube is 6 µL.

For any problem in method application you can contact AB ANALITICA technical support at: laboratorio@abanalitica.it .

12.2 DNA amplification

ATTENTION: The premix can be placed on more sponges, which are positioned one on top of the other.

For each sample, add to each premix tube:

Super AB Taq	0.2 µL
DNA	6 µL
V_{FINALE}	35 µL

ATTENTION: Add 2 drops of mineral oil (not included) to each premix if required by the thermocycler.

It is important to include in each experiment a negative control to monitor the contamination (add distilled water to the mix instead of extracted DNA) and a reference DNA (DNA of a undeleted male) that will show all the bands of the different markers.

Put the microtubes into the thermalcycler programmed as below:

1 cycle	94°C	5 min
40 cycles	94°C	1 min
	60°C	1 min
	72°C	1 min
1 cycle	72°C	7 min
storage	4°C	

12.2.1 Table 1: Lengths of the amplification products from the multiplex amplification

MX 1	bp	MX 2	bp	MX 3	bp
ZFX/ZFY	**495**	**ZFX/ZFY**	**495**	DBY	689
SRY	**472**	**SRY**	**472**	**ZFX/ZFY**	**495**
sY254	380	sY95	303	**SRY**	**472**
sY86	320	sY117	262	sY84	326
sY127	274	sY125	200	sY134	301
sY255	120			DFFRY	155

NOTE: The amplification of the ZFX/ZFY gene is the internal PCR control because the primers amplify an unique fragment found in both male and female DNA.

12.3 VISUALIZATION OF THE AMPLIFICATION PRODUCTS

12.3.1 Agarose gel electrophoresis

Prepare a 3% agarose gel:
Weigh 1.5 g of agarose and add it to 50 mL of 1X TAE.
Leave the solution on a magnetic stirring heater or in a microwave, until the solution becomes clear. (ATTENTION: keep the power at a low level in order to avoid the excessive production of bubbles).
Allow the gel to cool for a few minutes and then add 10 µL of 2.5 mg/mL Ethidium Bromide solution.

CAUTION: Ethidium Bromide is a strong mutagenic agent: Always wear gloves and preferably work under a chemical safety cabinet during the handling of this reagent or gels containing it.

Pour the gel into the appropriate gel casting tray, with the comb placed in and allow the gel to cool at room temperature or in a fridge until the gel becomes solid.
Remove the comb carefully (pay attention to not damage the gel's wells), transfer the tray into the electrophoresis chamber and pour the appropriate amount of 1X TAE buffer so that it covers the gel completely (about 1-2 mm over the gel surface).

12.3.2 Sample loading

For visualization of the amplification products, mix into a tube or directly on a parafilm layer:

2 µL Bromophenol blue *
10 µL Amplification product of each multiplex PCR
and
2 µL Bromophenol blue *
10 µL DNA Molecular Weight Marker*

NOTE:
Bromophenol Blue* and DNA Molecular Weight Marker * are included in code 04-23A only; if other loading buffers or molecular weight markers are used, refer to the manufacturer's instructions.

Load the mixture in the gel wells; switch on the power supply and set the voltage between 80-90 V.
Run the gel for about 2 hours, then place the gel on an UV transilluminator and analyze the results by comparing the size of the amplification products with the reference Molecular Weight Marker.

* DNA Molecular Weight Marker (Marker MW):

501- 489, 404, 331, 242, 190, 147, 111, 110, 67, 34x2, 26 bp.

(NOTE: In a 3% agarose gel the 501-489 bp bands usually are not clearly resolved and appear as an unique band; the 26 and 34 bp bands are sometimes too small to be visible in a 3% agarose gel (because of their low molecular weight).

NOTICE:
UV rays are dangerous for skin and, above all, eyes: always wear gloves and safety glass or make use of the protection screen of UV transilluminator.

13 INTERPRETATION OF THE RESULTS

The included controls should show the following results:

CONTROL	RESULTS	INTERPRETATION
Reference DNA	Presence of all the expected bands	The multiplex PCR amplification works correctly.
Negative control	Absence of bands	Absence of contaminations

Then the interpretation of the bands on agarose gel follows the table below:

RESULTS	INTERPRETATION
Absence of the ZFX/ZFY band Absence of some or all markers	Sample not amplifiable
Presence of the ZFX/ZFY band Presence all markers Presence of the SRY band	Amplifiable sample, without any deletion at the AZF locus, and presence of the testis determining factor.
Presence of the ZFX/ZFY band Absence of all markers Presence of the SRY band	Amplifiable sample, without AZF locus and presence of the testis determining factor (e.g XX-male).
Presence of the ZFX/ZFY band Absence of one or more markers	Amplifiabile sample, with deletions at the AZF locus.

If the results show that the sample is not amplifiable (absence of the band of the internal control ZFX/ZFY) the complete analysis should be repeated.
The parallel amplification of the SRY marker and the internal control ZFX/ZFY allows the monitoring of any female contamination.

MARKERS / INTERNAL CONTROL	NOT-DELETED XY MALE	XX MALE	XX FEMALE	DELETED XY MALE
AZF MARKERS	+	--	--	+ / --
ZFX / ZFY	+	+	+	+
SRY	+	+	--	+

In case one or more markers absent, it is suggested, as indicated in the guide lines, to repeat the amplification using the pair of specific primers for that marker with a simplex amplification.

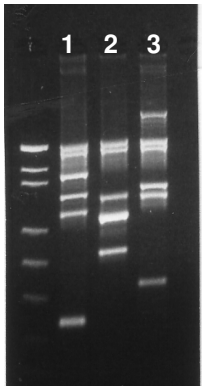

Fig 2.
3 % Agarose gel electrophoresis of the three multiplex PCR amplifications.

DNA Molecular Weight Marker
1. Multiplex I
2. Multiplex II
3. Multiplex III

The analysed patient is not deleted for any of the markers.

For any problem you can contact AB ANALITICA technical support:
e-mail: laboratorio@abanalitica.it
fax N +39 049-8709510.

14 TROUBLESHOOTING

1. Neither amplification products, nor reference DNA band

- TAQ polymerase was not correctly added to the premix

- Use pipettes and tips of suitable volumes (pipette range 0.2 - 2 µL) and suitable tips.
- Check visually that TAQ polymerase diffuses in the premix: this is easy because the enzyme is in dissolved glycerol that has a higher density.
- Alternatively, put the drop of TAQ polymerase on the tube wall, then centrifuge briefly.

- The thermalcycler was not programmed correctly.

Check the conformity of the thermalcycler program and the temperature profile in the instruction manual.

- The kit doesn't work properly

- Store the premixes, TAQ polymerase and reference DNA at -30°/ -20°C;

- Avoid repeated freezing and thawing of the reagents.

2. No amplification bands (or only for some markers), absence of the ZFX/ZFY band, but a good band for reference DNA

Possible problems during the extraction step:

Verify the following points and proceed as suggested:

- Verify that you followed very careful the manufactures's instructions of the extraction kit.
- Consult the "troubleshooting" section of the user manual of the extraction kit.
- Repeat the DNA extraction from a new sample.

3. Some inhibitors or other factors which interfere with amplification reaction are present.

1. The obtained DNA solution is not pure (the ratio A260/A 280 is low). Verify to have correctly followed the extraction protocol and repeat the extraction by starting from a new sample;

2. In the obtained DNA solution there is residual RNA (the ratio A260/A 280 is too high) which can be eliminated by introducing a digestion step with RNAase.

For any further problems contact AB ANALITICA's technical support at: laboratorio@abanalitica.it, fax (+39) 049-8709510, or tel. (+39) 049-761698.

15 DEVICE LIMITS

The kit can have reduced performances if the clinical sample is not suitable for this analysis (blood sample non properly stored or treated with heparin as anti-coagulant)

16 DEVICE PERFORMANCES

16.1 Specificity

The primers used in this method were studied in a way that they do not amplify repeated sequences in the chromosome or DNA sequences that have big nucleotidic differences among the population.
Primer sequence alignment in the most important databanks shows the absence of unspecific alignment. Moreover, experimental data (total absence of feminine DNA amplification) revealed that the primers used for each marker are Y-specific.

16.2 Diagnostic sensitivity

The use of a basic set of STS primers, as suggested in the European Guidelines enables the detection of almost all the clinically relevant deletions and over 95% of the deletions reported in literature in the three AZF loci.

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